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***published in***
Microbiology spectrum
2014

***DOI (link to publisher)***
10.1128/microbiolspec.PLAS-0030-2014

***document version***
Publisher's PDF, also known as Version of record

Microbiology Spectrum
American Society for Microbiology Press

# Plasmid-Encoded Iron Uptake Systems

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**ABSTRACT** Plasmids confer genetic information that benefits the bacterial cells containing them. In pathogenic bacteria, plasmids often harbor virulence determinants that enhance the pathogenicity of the bacterium. The ability to acquire iron in environments where it is limited, for instance the eukaryotic host, is a critical factor for bacterial growth. To acquire iron, bacteria have evolved specific iron uptake mechanisms. These systems are often chromosomally encoded, while those that are plasmid-encoded are rare. Two main plasmid types, ColV and pJM1, have been shown to harbor determinants that increase virulence by providing the cell with essential iron for growth. It is clear that these two plasmid groups evolved independently from each other since they do not share similarities either in the plasmid backbones or in the iron uptake systems they harbor. The siderophores aerobactin and salmochelin that are found on ColV plasmids fall in the hydroxamate and catechol group, respectively, whereas both functional groups are present in the anguibactin siderophore, the only iron uptake system found on pJM1-type plasmids. Besides siderophore-mediated iron uptake, ColV plasmids carry additional genes involved in iron metabolism. These systems include ABC transporters, hemolysins, and a hemoglobin protease. ColV- and pJM1-like plasmids have been shown to confer virulence to their bacterial host, and this trait can be completely ascribed to their encoded iron uptake systems.

## INTRODUCTION

Iron is one of the most important metals for life, as it is necessary for the proper functioning of proteins that mediate essential cellular processes such as DNA precursor synthesis, respiration, photosynthesis, and nitrogen fixation. Iron is one of the most abundant elements on earth; however, its bioavailability is very low. In the presence of oxygen, ferrous iron oxidizes to ferric iron that is poorly soluble at neutral pH. Additionally, free iron is toxic due to the formation of free oxygen radicals that can cause cell damage (1). Therefore, in biological systems the iron is complexed to keep it soluble and to reduce the toxicity of free iron.

To overcome the low availability of iron in their environment, bacteria have evolved specialized mechanisms to take up either the scarce soluble ions or to compete with iron-chelating complexes (2). In particular, pathogenic bacteria face extremely low iron concentrations due to the presence of high-affinity iron-binding proteins in the host that perform a dual function: they protect cells from the toxic effect of free iron while inhibiting bacterial growth (3). Competition for iron within the host is thus a critical factor in host-pathogen interaction (4).

Mechanisms for iron uptake range from direct binding of transferrin, lactoferrin, and heme by outer membrane receptors, to compounds with a high affinity for iron that strip the metal ions chelated to protein and complexes (5). To take up iron from the host iron-binding proteins transferrin and lactoferrin, bacteria express specific outer membrane receptors. Upon binding to the receptor, iron is released and transported through the receptor to the periplasmic space and subsequently

**Received:** 30 September 2014, **Accepted:** 1 October 2014, **Published:** 5 December 2014

**Editors:** Marcelo Tolmasky, California State University, Fullerton, CA, and Juan Carlos Alonso, Centro Nacional de Biotecnología, Cantoblanco, Madrid, Spain

**Citation:** Di Lorenzo M, Stork M. 2014. Plasmid-encoded iron uptake systems. *Microbiol Spectrum* 2(6):PLAS-0030-2014. doi:10.1128/microbiolspec.PLAS-0030-2014.

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to the cytoplasm (6, 7). Bacteria can also obtain iron from red blood cells via similar though more complex mechanisms. Hemoglobin and heme are released from hematocytes by the action of cytolysins and hemolysins. Receptors at the bacterial surface recognize the free heme or hemoproteins and transport heme to the periplasm. Once inside the cell, iron is released from heme (8). More elaborate heme acquisition systems secrete heme-scavenging molecules, the hemophores, which extract heme from host hemoproteins and deliver it to an outer membrane receptor for internalization (8).

Siderophore-mediated systems are the most studied iron uptake mechanism of bacteria. Siderophores are low-molecular-weight compounds that have a very high association constant for iron, up to $10^{50}$ (9). Most of these secondary metabolites are synthesized via a nonribosomal peptide synthetase mechanism (10). Siderophores are produced in the cytoplasm and then secreted to the extracellular space. Outside the cell, the siderophore competes for iron with iron complexes, such as the iron-binding proteins lactoferrin and transferrin in the host (11, 12). Iron-loaded siderophores are bound by cognate receptors, and iron is internalized via a specific uptake system in the membrane of the bacterium (13). The uptake of ferric-siderophores by the bacterium is initiated by a chance encounter of the bacterial outer membrane receptor with the iron-loaded siderophore.

In all cases, transport of iron across the membranes needs energy. In Gram-negative bacteria there is no energy generation in the outer membrane, so the energy required to cross it is provided by the inner membrane. The proton motive force of the inner membrane is transduced to the outer membrane receptors by the TonB complex. This complex consists of several proteins (TonB, ExbB, ExbD, and in some cases TtpC) that are always chromosomally encoded (14–17). Once the iron or iron complex is in the periplasm, periplasmic binding proteins, either soluble or membrane-bound through a lipid anchor, mediate delivery to a complex in the inner membrane that usually belongs to the ATP-binding cassette (ABC) transporter family. The ABC transporters consist of two inner membrane permeases and an ATPase that hydrolyzes ATP to energize transport of the ligand across the cytoplasmic membrane (18).

While outer membrane receptors are highly specific for the system they belong to, the rest of the proteins involved in transport into the cytosol are often interchangeable between different iron uptake systems (18). Once it is in the cytoplasm, iron can be used for immediate cellular needs or stored in bacterioferritins (19).

The gene products involved in iron uptake are tightly regulated by iron availability. In iron-replete environments the genes are repressed by the ferric uptake regulator (Fur) (20–22). Fur in complex with iron binds to specific DNA sequences at the promoter regions of iron-regulated genes and blocks transcription. When iron is limiting, Fur no longer contains iron and therefore cannot bind the DNA. As a consequence, Fur repression is released and transcription proceeds (22). In addition to Fur, expression of iron uptake genes is also controlled by numerous small RNAs as well as regulatory proteins and two component systems (23, 24).

Most bacteria have at least one iron uptake mechanism, although the presence of multiple systems is quite common. Although the majority of these systems are chromosomally encoded, some of them are plasmid-mediated (25). The most studied plasmids harboring iron uptake systems are the ColV plasmids, found mainly in *Enterobacteriaceae*, and the pJM1-type plasmids, found in *Vibrio anguillarum* strains. Interestingly, there is no homology between these two plasmids and the systems they carry (26). In this article, we describe these two plasmid types and their iron uptake systems.

## ColV PLASMIDS

ColV plasmids are large and belong to the IncFI incompatibility group. Plasmids in this group are heterogeneous in size (typically from 80 to 180 kb), genetic composition, ability to conjugate, and virulence properties (27).

These plasmids often possess more than one IncF replicon, but the replicons and their genetic organization are not always conserved across the different plasmids. The *repFIB* replicon is the most widespread among ColV plasmids, and it is located between the *sitABCD* and the *etsABC* regions (27–31). The *repFIB* replicon of pColV-K30 encodes two essential factors for replication: an initiator protein, RepI, and five 18-bp direct repeats (32–34). It has been proposed that upon binding to the direct repeats at the origin of replication, RepI facilitates the opening of the DNA helix, initiating replication. Furthermore, when bound to the origin, RepI also represses its own expression (35, 36). Another replicon is commonly found in proximity to the transfer region; while in most cases this is a *repFIIA* replicon, in plasmids pAPEC-1 and pAPEC-O1-ColBM a composite *repFIIA-repFIC* origin is present (28, 31). Some ColV plasmids also carry a replicon homologous to *repFIA* of plasmid F (37). In these instances, a genetic organization with the two replicons *repFIA* and *repFIB* flanking the aerobactin

system seems to be conserved even in non-ColV plasmids, such as *Escherichia coli* R plasmids (38).

ColV plasmids have long been associated with the virulence of extraintestinal pathogenic *E. coli*, ExPEC (29, 39). This group of pathogenic *E. coli* has acquired numerous virulence traits, which confer the ability to cause an infection outside the host intestinal tract. The ExPEC group includes human and animal pathogens that cause urinary tract infections, neonatal meningitis, and septicemia. Avian pathogenic *E. coli* (APEC) strains are also ExPEC, and ColV plasmids are a defining feature of the APEC pathotype (40). ColV plasmids have also been identified at high frequencies in strains of *Salmonella enterica* serovar Kentucky isolated from chickens (41, 42).

ColV plasmids owe their name to colicin V, a toxin that is active against *E. coli*, *Shigella*, and *Salmonella* strains. It was first described by Andre Gratia in 1925 (43) and later shown to be transferable and linked to F-type plasmids (44, 45). Colicin V, the first identified colicin, is a microcin, a low-molecular-weight peptide antibiotic secreted through a dedicated ABC transporter (46, 47). Colicin V exerts bactericidal activity only at the periplasmic face of the inner membrane, where it interferes with energy production by disrupting the membrane potential (48). Four plasmid-encoded genes (*cvaA*, *cvaB*, *cva*C, and *cvi*) are involved in colicin synthesis, export, and immunity (Fig. 1). In addition, two chromosome-encoded genes (*cvpA* and *tolC*) are required for production and secretion (46, 49). Colicin V is often found in association with colicin Ia (50). Plasmid pS88 is an example of a plasmid harboring colicin V and colicin Ia operons (51). Colicin Ia has the same mode of action as colicin V, but while the production of colicin Ia is induced under stress conditions, the expression of colicin V is regulated by iron (49, 52).

Other colicin-encoding plasmids have been identified among ExPEC and APEC, the ColBM plasmids. These plasmids have received less attention than the more widespread ColV plasmids, but recent studies have shown that the ColBM plasmids harbor virulence clusters similar to those present on ColV plasmids. Analyses of the available sequences of ColBM plasmids suggest that they have evolved from an ancestral ColV plasmid by insertion of the ColBM region within the ColV operon. In fact, high DNA homology between the plasmids pAPEC-O2-ColV and pAPEC-O1-ColBM is observed in the virulence clusters, and 67% of the predicted proteins of pAPEC-O1-ColBM are also found on pAPEC-O2-ColV (28). These two plasmids also have nearly identical transfer regions and possess *repFIB* and *repFIIA* replicons (28). Alternatively, transfer of the colicin V genes and associated virulence factors onto ColBM plasmids could have occurred (53).

The ColBM region harbors the ColB and ColM operons. These two operons encode colicin B and colicin M, respectively. Each operon consists of a structural gene for the colicin (*cba* in ColB and *cma* in ColM) and an immunity gene (*cbi* in ColB and *cmi* in ColM) encoding a protein that binds and inactivates the colicin. Colicin B is a pore-forming colicin and has a mode of action similar to colicin V (49), while colicin M, the smallest known colicin, inhibits peptidoglycan biosynthesis (54). Interestingly, both colicins, as well as colicin V and Ia, gain access to sensitive cells via TonB-dependent receptors: the enterobactin receptor FepA for colicin B, the ferrichrome receptor FhuA for colicin M, and the catechol receptor Cir for colicin V and colicin Ia (47, 55). The use of iron-regulated receptors for internalization and their iron-regulated expression show the intimate link between colicins and iron. Colicin-producing cells might have an advantage in iron-limited environments, since development of resistance in sensitive cells might occur at the cost of iron uptake efficiencies since it could mean the loss of TonB-dependent receptors (47).

Despite their name, it was shown early on that the virulence phenotype conferred by the ColV and ColBM plasmids is not colicin-mediated (56, 57). Several other determinants that are present in different combinations in the various plasmids of the ColV group have been shown to contribute to virulence. ColV plasmids harbor genes that encode serum resistance, adherence, resistance to chlorine and disinfectants, bacteriophage resistance, hemagglutination properties, and several iron acquisition systems (29). The presence of several clusters involved in iron metabolism is not surprising when considering the extraintestinal lifestyle of ExPEC strains, where iron is one of the main limiting factors for colonizing bacteria.

## Aerobactin Iron Uptake System

The first ColV iron uptake system identified was the one that codes for the hydroxamate siderophore aerobactin and its outer membrane receptor (58). The aerobactin system is, together with colicin V, the hallmark of ColV plasmids. Aerobactin was first discovered in the supernatant of *Aerobacter aerogenes* 62-I and was later shown to be encoded by plasmid pSMN1 (59, 60). Even though they are both plasmid-encoded, the aerobactin cluster of pSMN1 shares only a weak homology with the genes encoding the aerobactin system found on the ColV plasmids (61). These two genetically distinct systems

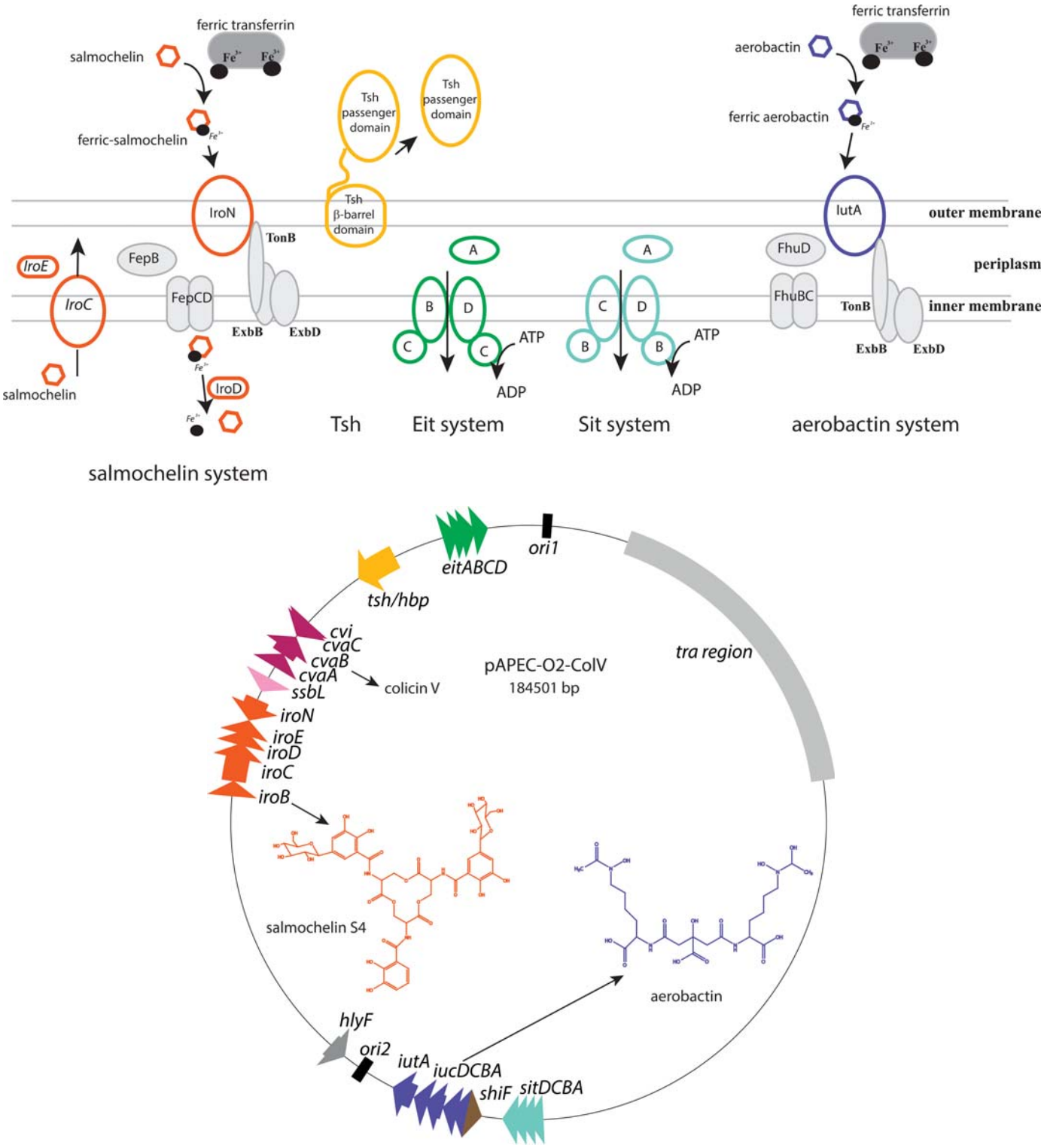


**FIGURE 1** Schematic representation of a ColV plasmid (30) showing all open reading frames related to iron uptake, their function, and their membrane localization when relevant. Each system is color-coded. The *tra* region is shown as a gray box, and the origins of replication are shown as black boxes. Structures of the two siderophores aerobactin and salmochelin S4 are shown within the plasmid. doi:10.1128/microbiolspec.PLAS-0030-2014.f1

nonetheless produce siderophores with identical structures. Interestingly, the aerobactin cluster is also found on non-ColV R plasmids of *Salmonella* and *E. coli* (38, 60, 62, 63). In contrast to the case of pSMN1, the aerobactin sequences found on ColV and *E. coli* and *Salmonella* R plasmids are conserved and linked to the *repFIB* replication region (37, 62). It is worth noting that the origin of replication of plasmid pSMN1 is not of the IncFI incompatibility group (61), which suggests a linkage between the *repFIB* origin of replication and the aerobactin cluster found on ColV and R plasmids. They could constitute a virulence factor-replication unit that ensures perpetuation of the aerobactin genes even in the event of a deletion (37). The aerobactin system was also found in close proximity to the *repFIB* replicon in *Chronobacter* virulence plasmid and in a plasmid isolated from a sewage treatment plant (64–66).

The aerobactin genes in ColV and *Salmonella* R plasmids are flanked by the inverted IS*1* sequences that could contribute to the spread of this system either by homologous recombination with other IS*1* sequences or by transposition (37, 67, 68). The increased mobility conferred by the flanking IS*1* sequences could explain the occurrence of the aerobactin system also on chromosomes of *E. coli*, *Salmonella*, and *Shigella* strains (60, 69–73). The presence of a colicin system within the pathogenicity island (PAI) of *Shigella flexneri* upstream of the aerobactin genes further supports the hypothesis of a ColV origin for the chromosomal systems (73). On the other hand, the absence of flanking IS*1* sequences both on plasmid- and chromosome-encoded aerobactin systems as well as some sequence divergence, might argue against an IS*1*-mediated mechanism of dissemination and a ColV origin (74, 75).

The gene cluster of the aerobactin system consists of five open reading frames, *iucABCDiutA* (Fig. 1) (76). The products of the *iuc* genes are involved in the biosynthesis of aerobactin from lysine and citrate. The citrate molecule serves as a central linker for two molecules of $N^6$-acetyl-$N^6$-hydroxy lysine. These molecules are produced from lysine in two steps. In the first step, the IucD monooxygenase utilizes molecular oxygen to generate $N^6$-hydroxy lysine. In the subsequent step, the acetylase IucB *N*-acetylates the hydroxy lysine using acetyl-CoA (77). The final coupling of two molecules of $N^6$-acetyl-$N^6$-hydroxy lysine to citrate is catalyzed by the two synthetases IucA and IucC. Each synthetase assembles one modified lysine molecule onto the citrate, forming an amide bond between the α-amino group of lysine and the carbon of one of the carboxyl groups of citrate (78). The last gene of the cluster, *iutA*, encodes the outer membrane receptor for ferric aerobactin that functions in a TonB-dependent manner (79). Once in the periplasm, aerobactin is bound and transported across the inner membrane by the generic chromosomally encoded hydroxamate transport system, which consists of a periplasmic binding protein, FhuD, and inner membrane permeases, FhuBC (80–82). The clusters coding for the TonB complex and the hydroxamate transport system are both located on the chromosome (83).

Like other iron uptake systems, expression of the aerobactin cluster is negatively regulated by Fur and the iron state of the cell (84). Fur binds in an iron-dependent manner to multiple sites within a large region of the aerobactin operon promoter (85).

Several pieces of evidence point to aerobactin as a virulence factor. A large percentage of *E. coli* strains isolated from human extraintestinal infections tested positive for aerobactin, while only one-third of normal fecal isolates carried genes of the aerobactin system (86, 87). Among avian *E. coli* isolates, only the most virulent harbor the aerobactin genes, while these same genes are absent from nonvirulent and nonlethal isolates (88, 89). Furthermore, aerobactin genes were expressed during infection, and defined deletion mutants in biosynthetic and transport genes resulted in decreased virulence in a chicken infection model (90–92). In whole-plasmid transcriptional studies, an additional gene, *shiF*, was upregulated during growth in human serum and urine and in a neonatal rat sepsis model (93, 94). The *shiF* gene has been found to be conserved upstream of the aerobactin cluster, in some cases in association with another gene, *viuB* (51, 64–66). Based on their homology, the products of these two genes have been proposed to be part of the aerobactin iron uptake system (64–66, 93).

## Salmochelin Iron Uptake System

The salmochelin iron uptake system, encoded on the chromosome of *Salmonella* species and of several uropathogenic *E. coli* (UPEC) (95–98), has been recently associated with the ColV plasmids of APEC strains (90, 92, 99, 100) as well as with non-ColV transmissible plasmids of UPEC and *Klebsiella* strains (101, 102).

Salmochelins are catechol siderophores that consist of enterobactin glucosylated at one or two of the 2,3-dihydroxybenzoyl (DHB) rings (103). Salmochelin S4 (Fig. 1), the most common form, has the macrolactone of enterobactin intact, while this is hydrolyzed in salmochelin S2, where the unglycosylated DHB-serine moiety is located at the C-terminal end. Salmochelins S1 and SX are, respectively, the dimer DHB(gucosyl)-seryl-DHB-serine and the monomeric DHB(glucosyl)-serine

molecule. All these salmochelin forms have been isolated from *S. enterica* growth medium, but as in the case of enterobactin, these linear forms are probably the degradation products of salmochelin S4, the key metabolite (103). Since the substrate for salmochelin S4 synthesis is enterobactin itself, genes of the enterobactin cluster are also essential for salmochelin biosynthesis (103, 104). Glycosylation of enterobactin results in a siderophore with reduced hydrophobicity that is no longer recognized and bound by the host protein siderocalin and serum albumin (103, 105, 106). Perhaps immunologic pressure in serum resulted in a selective advantage for strains harboring salmochelin over those producing enterobactin.

Genes coding for salmochelins and the outer membrane receptor were first identified in the chromosome of *Salmonella* serovar Typhimurium in the Fur-regulated locus, *iroA* (96). The *iroA* locus consists of two divergently transcribed sets of genes, *iroBCDE* and *iroN* (95).

IroN is an outer-membrane protein with homology to TonB-dependent receptors. IroN is the receptor for salmochelins, as shown by uptake experiments with radiolabeled $^{55}$F-salmochelin and growth promotion assays (107). Salmochelin uptake can also be facilitated by the enterobactin receptor FepA and the Cir receptor in *Salmonella* (107). On the other hand, IroN can transport, besides salmochelins, other catechol siderophores, such as enterobactin and corynebactin (102, 108). An additional role as an internalization factor has been proposed for IroN during invasion of urothelial cells by ExPEC strains (109).

The *iroB* gene is the only one in the *iroA* cluster involved in salmochelin biosynthesis (Fig. 1). IroB encodes a glucosyltransferase that glucosylates the DHB rings of enterobactin using UDP-Glc as the glycosyl donor. A remarkable characteristic of IroB is the ability to catalyze formation of a C-C bond during enterobactin glucosylation. In this regard, IroB differs from other glycosyltransferases, such as those identified in the antibiotics vancomycin and ramoplanin, which glycosylate oxygen atoms (104).

The *iroC* gene encodes a four-domain ABC exporter similar to eukaryotic multidrug resistance transporters (Fig. 1). IroC is involved in salmochelin S4 secretion rather than in the uptake of ferric-salmochelin, as shown by deletion of the *iroC* gene (110, 111).

The last two genes in the *iroBCDE* operon encode two proteins, IroD and IroE, which are predicted to have hydrolytic activity. IroD is a cytoplasmic protein similar to Fes, the esterase of the enterobactin system. IroE is instead predicted to be a periplasmic esterase. The function of these two esterases in the salmochelin system is still not well established as well as their substrate specificity. Most likely, IroD is the esterase responsible for release of iron from ferric-salmochelin into the cytoplasm. IroE acts in the periplasm and might be more important for export or processing of cyclic salmochelins prior to release (99, 110, 112).

Besides the *iroBCDE* and *iroN* plasmid-encoded genes, the entire chromosomally encoded enterobactin cluster is required for salmochelin biosynthesis and transport (113). In addition, the chromosomally encoded TonB complex is needed to transport salmochelin across the outer membrane.

It has been shown that salmochelin is required for full virulence in a chicken infection model (90). Mutations in the salmochelin system slightly affect virulence, while mutations in multiple iron uptake systems result in a dramatic decrease of chicken tissue colonization (91, 114). This is probably due to the high redundancy of iron uptake systems in pathogenic strains. Levels of salmochelin produced during infection of host tissues are similar to those produced in iron-poor culture medium (110). Although salmochelin levels are lower than the aerobactin levels, both *in vitro* and *in vivo*, production of salmochelin *in vivo* further supports a role in virulence for this siderophore. Transcriptional studies in human serum and urine point to a similar role of the salmochelin system found on plasmid pS88 of an *E. coli* strain that causes neonatal meningitis (93). Interestingly, in the same study a gene, *ssbL*, was identified that displayed the highest upregulation *in vivo*. The *ssbL* gene shows a strong association with the *iro* cluster (115). The *ssbL* gene is involved in the shikimate pathway, and its product has been proposed to enhance production of salmochelin by boosting the catechol metabolic pathway (115).

## SitABCD Iron Transport System

Operons encoding the SitABCD system have been found on the reference colicin V plasmid pColV-K30 and on ColV-like plasmids of APEC strains (28, 30, 31, 116, 117).

The SitABCD system is an ATP-binding cassette (ABC) transporter of divalent metal cations, originally identified on the chromosome of serovar Typhimurium in *Salmonella* PAI1, SPI1 (118). As in typical ABC transport systems, the *sit* genes encode a periplasmic protein, SitA, that binds the ligand and transfers it to the inner membrane permeases, SitC and SitD (Fig. 1). SitB, the ATPase of the system, catalyzes hydrolysis of ATP to energize transport across the cytoplasmic membrane of the ligand (119).

The *sitABCD* operon is expressed *in vivo* during the systemic stages of *Salmonella* serovar Typhimurium infection in mice. A mutation in the *sit* locus resulted in a defect in virulence of the mutant strain, although it was subtle, probably due to the presence of redundant systems for iron and manganese uptake (120). After its initial identification as a siderophore-independent iron ABC transporter, the Sit system was shown to be involved in manganese and ferrous iron transport from the periplasm to the cytoplasm (116, 121, 122). The ability of the SitABCD transport system to transport manganese might enhance resistance to oxidative stress and survival in extraintestinal tissues (123).

In APEC strains, *sit* genes are highly associated with virulence in 1-day-old chicks, and there is a prevalence of this system in virulent isolates when compared with fecal commensals (40, 123). Interestingly, although *sit* genes are also found on the chromosome of some *E. coli* strains, the *sitABCD* operon in APEC strains is mainly plasmid-encoded (124).

## EitABCD System

An additional ABC transport system, EitABCD, has been found on pAPEC-O2-ColV and on pAPEC-O1-ColBM plasmids (28, 30). EitABCD is similar at the protein level, with an iron transport system of the plant pathogen *Pseudomonas syringae* (30). The functionality of this ABC system in iron uptake has yet to be determined. The homology between the Eit and Sit ABC transporters present on the pAPEC-O2-ColV plasmid is very low, and the functional genes also differ in the order within the clusters (Fig. 1). While the SitABCD system has been shown to be a metal ion transporter, EitABCD shares similarity with other ABC transporters involved in siderophore transport.

Other ColV plasmids sequenced so far do not harbor the *eitABCD* cluster, while this system is found on non-ColV *repFIB* and *repFII* plasmids (29). Interestingly, these two replicons are those harbored by several ColV plasmids. The *repFIB* plasmids, found in 97% of 229 *Chronobacter* spp., emerging neonatal pathogens, harbor the Eit system. The *eit* gene arrangement in the *Chronobacter* plasmids, pESA3 and pCTU1, differs from the typical single-operon *eitABCD* found in several enteric pathogens. In these *Chronobacter* plasmids the genes are organized as two divergent operons, *eitABC* and *eitD* (64). Expression of these *eit* operons does not seem to be regulated by iron despite the presence of putative Fur boxes upstream of the *eitA* and *eitD* genes (65). IncFII plasmids harboring the *eitABCD* cluster are instead found in APEC and *Klebsiella* strains (125–127).

The prevalence of *eit* genes among ExPEC of human and avian origin is low, which could indicate a recent acquisition of these genes by ExPEC strains (126).

## Temperature-Sensitive Hemagglutinin Tsh

The Tsh protein of APEC strain χ7122 was characterized as a hemagglutinin with a proteolytic domain expressed at lower temperatures (128). This hemagglutinin was the first identified member of an expanding subclass of the IgA protease family of autotransporters present in *Shigella* and numerous pathotypes of *E. coli* (129). Autotransporters consist of three functional domains: a sec-dependent amino-terminal leader sequence, a passenger domain, and an outer membrane-associated carboxy-terminal β-barrel domain (see Fig. 1) (129). The latter domain mediates secretion of the passenger domain, the extracellular or surface-secreted mature protein. The 106-kDa passenger domain of Tsh contains a serine protease motif whose function could not be demonstrated (130). Tsh is almost identical to Hbp (only two amino acid differences), an autotransporter from an *E. coli* clinical isolate from a wound infection. Hbp was shown to specifically degrade human hemoglobin and bind heme and it was proposed to be part of a hemophore-dependent heme acquisition system (131). A similar role was speculated for the other members of the Tsh family (131).

The *tsh* genes as well as the *hbp* genes are often located on plasmids (131, 132). In a screening study of 300 avian *E. coli* isolates, the *tsh* gene was more prevalent in high-lethality isolates, where it was always plasmid-encoded (132). In the same study, it was shown that the majority of the plasmids harboring *tsh* were of the ColV-type, while a restricted minority was not (132). The association of the *tsh* gene with large plasmids containing the colicin V gene cluster is confirmed by the growing number of complete sequences of ColV plasmids, although not all of the ColV plasmids harbor the *tsh* gene (117). The *tsh* gene has also been found within PAI III of UPEC strain 536 together with the *iro* cluster and a putative heme receptor (98). The different distribution of *tsh* compared to the widespread aerobactin system supports their association with virulence of APEC strains; it is worth noting that although the *tsh* gene is associated with high lethality, the highest level of virulence seems to require the aerobactin siderophore and not Tsh (89). In agreement with this is the minor role of iron acquisition through heme during infection, which is confirmed by studies with mutant strains in a chicken infection model (91). On the other hand, Tsh might have a role in the air sacs, as supported by infec-

tion studies and expression of the gene in the air sacs (90, 132). Other iron uptake systems, such as aerobactin and salmochelin siderophores, might be more important in deeper tissues (90).

## Hemolysin

Another gene involved in iron uptake and associated with ColV plasmids is the *hlyF* gene (Fig. 1). This gene has been found in the majority of the APEC ColV plasmids and in several ExPEC strains (29, 31, 133). The *hlyF* gene, its product HlyF, and its mode of action still need to be characterized. HlyF has hemolytic activity, but its amino acid sequence shows no significant homology to HlyA, the RTX hemolysin of several *E. coli* pathotypes, or to HlyE, a more recently identified hemolysin distinct from HlyA (134–137). Besides HlyF, the HlyA hemolysin is associated with plasmids. In fact, while the *hlyCABD* operon is found on the chromosome in UPEC strains, large plasmids harbor hemolysin clusters in human enterohemorrhagic *E. coli* (EHEC) strains and enterotoxigenic, shigatoxigenic, and enteropathogenic *E. coli* strains isolated from animals (138–141). The HlyA hemolysin found in EHEC, the enterohemolysin, is distinct from the HlyA hemolysin found in the other *E. coli* pathogroups, the α-hemolysin (134, 137). Plasmids of EHEC strains are, like ColV plasmids, F-like plasmids, and they share a similar backbone for what concerns origins of replication and transfer region, although the majority of EHEC plasmids are transfer-defective (29, 142, 143). On the other hand, plasmids harboring the genetic determinants for production of α-hemolysin were found to be heterogeneous also in their incompatibility group (144, 145). DNA hybridization experiments indicate that plasmid-encoded α-hemolysin and enterohemolysin have evolved separately in EHEC and enteropathogenic strains, although they might exert the same function (141, 146). On the other hand, the high similarity at the sequence level of α-hemolysin operons present on plasmids in enterotoxigenic, shigatoxigenic, and enteropathogenic strains indicates a common origin for these genes (138, 146).

The *hlyA* gene encodes the pro-hemolysin that is converted to its active form by the product of the *hlyC* gene by transfer of two fatty-acyl residues onto pro-HlyA. A type-I secretory complex comprising the ATP-binding cassette HlyB, the membrane-fusion protein HlyD, and the outer-membrane protein TolC secrete the active HlyA as well as the pro-HlyA. HlyA interaction with the target cell membrane occurs in two steps: the α-hemolysin absorbs onto the membrane in a reversible manner that is followed by irreversible insertion within the membrane. Once HlyA is inserted in the membrane, oligomerization occurs, leading to pore formation and cell lysis (147).

The role of hemolysins in iron uptake is still under debate. It has been proposed that secretion of hemolysins and cytolysins can quickly increase the local concentration of free heme and hemoglobin. This proposal is supported by indirect evidence such as hemolysin expression often repressed by iron and the presence of heme transport systems in strains with hemolytic activity (134, 148, 149). The presence of *hlyF* together with the *tsh* gene on the same plasmid further supports the possibility of their products acting as a hemophore-dependent heme acquisition system in combination with a chromosomally encoded heme receptor, such as ChuA. Besides colocalization in some cases, *hly* genes and *chuA* share a positive regulator, as can be expected for genes that function in the same system (147, 150).

## Heterogeneity of ColV Determinants

As proposed by Waters and Crosa (27), a "constant" and a "variable" region, together spanning a 94-kb cluster of putative virulence genes, can be distinguished within the ColV PAI. The constant region contains the *repFIB* replicon, the aerobactin operon, the Sit iron and manganese transport system, a putative outer membrane protease gene, *ompT*, the hemolysin gene, *hlyF*, a novel ABC transport system known as Ets, the salmochelin siderophore system, and *iss* that encodes serum resistance (30). The temperature-sensitive hemagglutinin gene *tsh* and another novel transport system known as Eit are part of the variable portion, as they are less frequently associated with ColV plasmids than are the genes in the constant region (29). The divide between the two regions appears to be within the *colV* operon, with a clear higher cooccurrence of genes within the constant region (about 60%) than the 26% for genes of the variable region (30).

From the increasing number of large plasmid complete sequences, a remarkable high nucleotide similarity (>95%) in the common regions of ColV plasmids is becoming evident. This suggests that ColV plasmids have evolved by adding and deleting large blocks of DNA, and these rearrangements are most likely the product of IS-mediated recombination events that affect the core as well as the noncore regions. As a consequence, a great heterogeneity in gene content is found within the ColV plasmid group (29).

As a result of frequent recombination events, widespread diversity in the noncore components is found in the sequenced plasmids, none of which are identical to

each other with regard to gene content. In extreme cases, plasmids can lack the entire variable region. There is also an example of a strain harboring the constant region on one plasmid and part of the variable region (the *eit* operon) on another (31, 51, 126).

IS-mediated recombination events may also account for the occurrence of some of the ColV plasmid components on non-ColV plasmids or on the chromosome of *E. coli*, *Shigella*, *Salmonella*, and possibly other bacterial strains. It is not yet clear if this transfer of genetic information proceeds from plasmid to chromosome or from chromosome to plasmid. The fact that in *S. flexneri* PAI SHI-2 harboring the aerobactin system also encodes immunity to colicins I and V suggests that the aerobactin genes found in SHI-2 originated from a ColV plasmid (73). In contrast, it has been proposed that ColV plasmids function as transposon traps for virulence-related determinants, pointing to a flow of genetic information to the plasmid rather than from it (131).

Aerobactin is also found on *Klebsiella* high-molecular-weight plasmids, and the presence of this system has been associated with virulent isolates (75). Although the system found in *Klebsiella* plasmids shows homology with the ColV genes, a certain degree of divergence and the absence of IS*1* insertion sequences downstream of the genes in *Klebsiella* might indicate different origins of the two systems (75).

Genes coding for aerobactin, salmochelin, and the Sit systems have been found on non-ColV plasmids of *E. coli*, *Salmonella*, and *Klebsiella* that are often associated with antibiotic resistance genes (38, 51, 62, 75, 102, 151). Occurrence of resistance determinants on ColV plasmids from different bacterial hosts has also been reported (29, 41, 42). The high degree of conservation of these resistance elements and their presence on unrelated plasmids suggest a shared horizontal resistance gene pool available to bacteria from different environments (152). The association of drug resistance determinants with virulence plasmids is quite worrisome since it may enhance pathogenicity in environments where antibiotics are used.

## pJM1-TYPE PLASMIDS

Plasmids of the pJM1 type are only found in the bacterium *V. anguillarum*, and more specifically, only in serotype O1 strains of this bacterium. *V. anguillarum* is the causative agent of vibriosis in fish, a highly fatal hemorrhagic septicemic disease (153). Several studies showed that *V. anguillarum* strains harboring pJM1 have a reduced infectivity in the absence of the plasmid (153–158). Plasmids of the pJM1 family range between 65 and 70 kb, and their difference in size is mainly due to the number of insertion sequences present. Plasmids in this group are highly similar, as shown in independent studies using restriction length polymorphism, where only a few patterns were identified among numerous different isolates from the United States, Japan, Spain, and Denmark (159–162).

To date, three complete annotated sequences of pJM1-type plasmids are available: the pJM1 plasmid from *V. anguillarum* 775 (65,009 nucleotides) and pEIB1 from *V. anguillarum* MVM425 and plasmid M3 from *V. anguillarum* M3, which are both 66,164 nucleotides (161, 163, 164). These plasmids are highly conserved and differ only in few nucleotides over the 65 kb they have in common.

The vast majority of the genes encoded on the pJM1-type plasmids are involved in iron uptake, either directly or indirectly (Fig. 2). The remaining genes code for proteins involved in plasmid replication, insertion sequences, and those for which no function can be assigned based on their homology (161, 163, 164).

### Replication and Transfer

The origin of replication was first identified on the pJM1 plasmid and later was further characterized on pJM1 and the pJM1-type plasmid pEIB1 (164–166). A 1,400-bp Sau3A fragment from pEIB1 was identified that can replicate in *E. coli* and *V. anguillarum* as well as in other *Vibrio* species such as *Vibrio alginolyticus*. The region contains replication features such as DnaA binding sites and inverted repeats (164). Recombinant plasmids consisting of different pJM1 fragments containing this origin of replication (ori1) and a resistance marker could replicate in a plasmid-less derivative of *V. anguillarum* 775 but resulted in different copy numbers (166). However, in *E. coli* all fragments tested had similar copy numbers, in all instances higher than in *V. anguillarum*. Hence, the pJM1 plasmid ori1 has some kind of copy number control that only functions in *V. anguillarum* (166). In the same study, it was shown that DnaA is not required for replication of pJM1, in contrast with the results obtained with the pEIB1 plasmid (164, 166). Interestingly, deletion of ori1 resulted in a pJM1 derivative still able to replicate, indicating the presence of a second origin of replication (ori2). Ori2 is located at ORF25 (Fig. 2), but unlike ori1, it cannot replicate in *E. coli* (164, 166).

Genes such as those encoding DnaB, DnaC, and DnaG, which are needed for DNA replication, are provided by the chromosome as well as a polymerase since

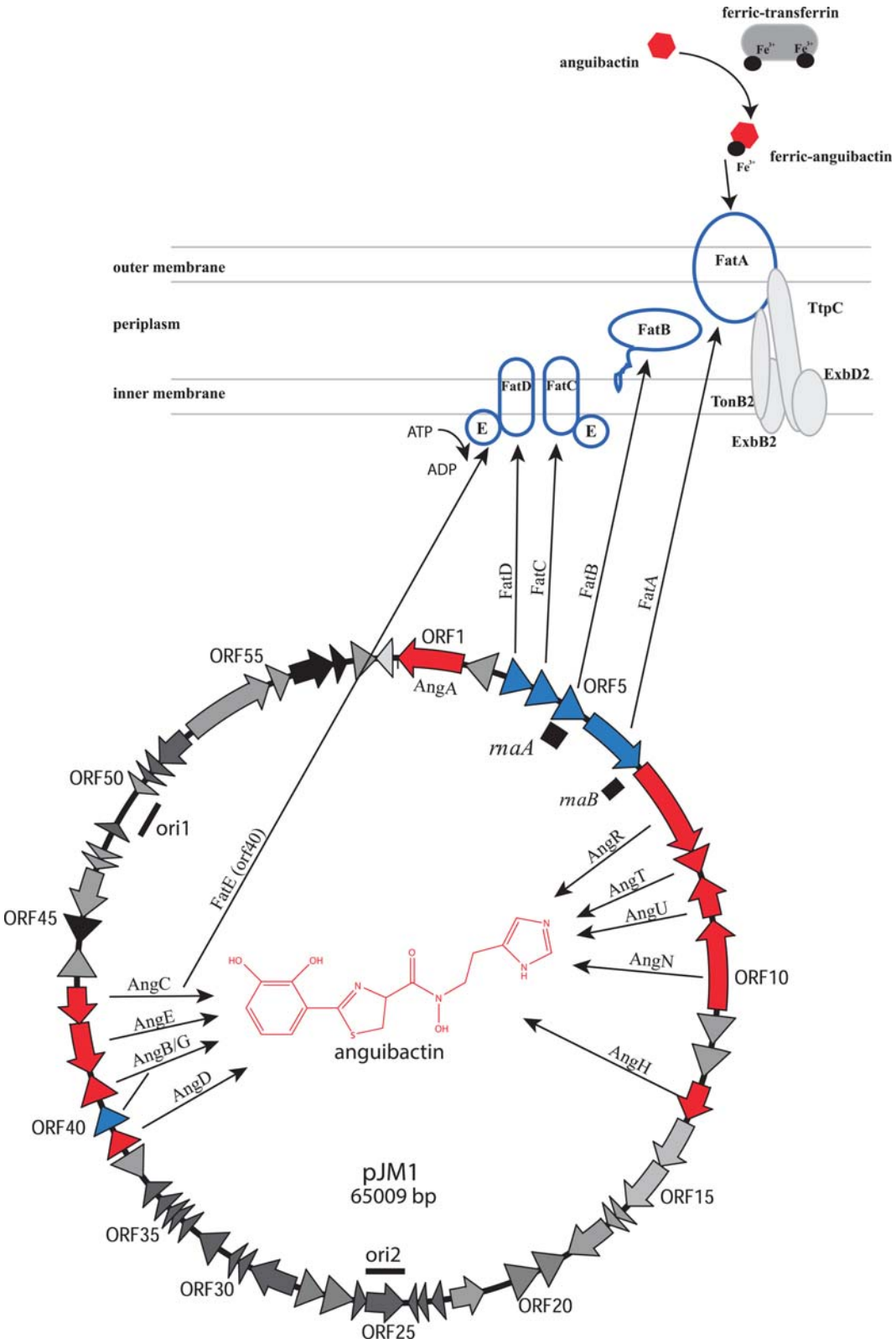


**FIGURE 2** Schematic representation of the pJM1 plasmid showing all open reading frames, the structure of anguibactin, and the transport proteins in the membranes. Genes that are involved in siderophore synthesis are shown in red; those involved in transport are blue. Black boxes indicate the location of the antisense RNAs. The shaded proteins in transport are chromosomally encoded. Location of the origins of replication is indicated by a black line. doi:10.1128/microbiolspec.PLAS-0030-2014.f2

no polymerase is encoded by the plasmid (161, 166). The incompatibility group for both replicons is unknown, and no homology with known origins of replication has been found. Ori1 is compatible with plasmids such as pUC18 (pMB1), pKA3 (pSC101), and p15A in *E. coli* (164). On the other hand, conjugation into *V. anguillarum* of some plasmids was prevented by the presence of plasmids of the pJM1 group (167). Singer and colleagues hypothesized that the lack of transconjugants in their study resulted from the presence of a restriction-modification system on pJM1-type plasmids (167). From the several complete sequences of pJM1-type plasmids available, it is evident that no restriction-modification system is present. An alternative explanation for the lack of transconjugants could be incompatibility between the replicon of the tested plasmids and one of the origins of replication of pJM1. The fact that ultraviolet treatment restored plasmid introduction by conjugation could then be explained by a mutation in the incompatible origin.

No transfer genes have been identified in the available complete sequences of pJM1-type plasmids (161, 163, 164). The lack of *tra* genes might explain the restriction of these plasmids to serotype O1 strains of *V. anguillarum* (168).

## Iron Uptake System

*V. anguillarum* 775, the prototype serogroup O1 strain, secretes a siderophore, a low-molecular-weight high-affinity iron-binding compound (169). This siderophore, called anguibactin, is linked to the plasmid, since siderophore production is abolished in a strain lacking the plasmid (157, 170, 171). In addition, the proteins needed for the transport of the ferric-siderophore across the membranes are also located on the plasmid (170–172). Fish infectivity studies showed that both the siderophore and the transport system are needed for infection. In strains harboring only the transport genes, establishment of an infection and replication in the bloodstream can occur only in the presence of strains producing the siderophore (158).

## Anguibactin Biosynthesis

The siderophore anguibactin is produced via a nonribosomal peptide synthetase mechanism. Nonribosomal peptide synthetases (NRPSs) catalyze peptide bond formation in the absence of an RNA template (10). Via this mechanism non-amino acid substrates, such as carboxylic acid, can also be incorporated. NRPSs are multimodular, and the order of the modules determines the order of the amino/carboxilic acids in the final product. A minimal NRPS consists of an adenylation domain (A) to activate the substrate, a peptidyl or aryl carrier domain (PCP/ArCP) to tether the activated substrate, and a condensation domain (C) to form a peptide bond between two activated substrates (173). In addition, there are domains that can modify the substrates, bringing even more variability to the peptide structure. In the case of antibiotic synthesis these modules are often located on one or two large polypeptides, while in the case of siderophore biosynthesis these modules are scattered over multiple proteins.

The building blocks of anguibactin are *N*-hydroxyhistamine, L-cysteine, and the carboxylic acid dihydroxy benzoic acid (DHBA) (169). All enzymes needed for the production of anguibactin are encoded on the pJM1- and pJM1-type plasmids (Fig. 2) (161, 163, 164). Genes for the biosynthesis of the anguibactin precursor DHBA, *angB* and *angC*, are also carried by the plasmid, with the exception of *angA*, for which only a truncated nonfunctional gene is present on the plasmid. As a consequence, for DHBA production, a functional AngA protein must be encoded on the chromosome. In some *V. anguillarum* strains, the chromosomal *angA* gene is present in a cluster with the other DHBA biosynthetic genes since these strains still produce DHBA when cured from the plasmid (174, 175). In the case of *V. anguillarum* 531A, curing of the plasmid abolishes DHBA production, pointing to an incomplete chromosomally encoded DHBA cluster (176). The plasmid also carries the genes for the synthesis of *N*-hydroxyhistamine from histidine. The *angH* and the *angU* genes encode, respectively, a histine decarboxylase and a histamine monooxygenase. Mutations in *angH* or curing of the plasmid abolished histamine production, indicating that there are no chromosomal copies of this gene (177, 178).

The synthesis of anguibactin begins with activation of DHBA by the adenylation domain of AngE. Activated DHBA is then tethered to the ArCP domain of AngG to further continue its assembly in the anguibactin molecule (100, 175, 176, 179). The *angG* gene is transcribed from the same promoter as *angB* and can be found in one polypeptide with AngB but also as a separate polypeptide (176). Tethering of activated substrates to ArCP and PCP domains of NRPSs occurs at a phosphopantetheinyl arm. This arm is posttranslationally added by AngD to a conserved serine residue within the ArCP and PCP domains (180).

L-cysteine is activated by the A domain of AngR and tethered to the PCP domain of AngM (181). The first amide bond forms between the DHBA tethered onto the ArCP domain of AngB and the amino group of the cysteine loaded on the PCP domain of AngM. This step

is followed by cyclodehydration of cysteine to a thiazoline ring. Both steps are mediated by the Cy domains of AngN (182). The final product, anguibactin, is then released by transfer of the dihydroxyphenyl thiazolyl intermediate from the PCP domain of AngM to the secondary amine of hydroxyhistamine in a reaction that requires the activity of the C domain of AngM (181). DHBA and histamine are dedicated metabolites for the synthesis of anguibactin, while L-cysteine is the only proteinogenic amino acid of the siderophore. Anguibactin has a quite compact structure, with three different chemical groups, all possessing iron-chelating properties (183). Furthermore, anguibactin belongs to the hydroxamate as well as the catecholate siderophores due to the presence of both functional groups.

## Transport

The uptake of anguibactin (Fig. 2) initiates with the binding of the ferric-siderophore to the outer membrane receptor FatA (184–187). FatA is a typical outer membrane receptor containing 22 antiparallel beta strands that form the barrel. It has a plug domain that blocks the open channel and extracellular loops that are needed for the initial binding to the siderophore (187). FatA then facilitates the transport over the outer membrane via an energy-dependent mechanism that requires the TonB system (16, 188). The genes of the TonB system are encoded on the chromosome (16, 188). *V. anguillarum* has two TonB systems, and both can transduce the energy to FatA. However, one of the two systems is much more efficient in the transport of anguibactin and requires an additional protein, TtpC, in the complex (16, 188). Once in the periplasm, ferric-anguibactin is bound by FatB, a lipoprotein anchored to the outer leaflet of the inner membrane (189–191). Anchoring of the periplasmic binding protein is not essential since transport still occurs when the lipid moiety is removed (191). However, transport is abolished in the absence of the FatB protein. From the periplasmic binding protein FatB, the ferric-siderophore is transferred to FatC and FatD. FatC and FatD form a typical ABC transporter and are both essential for internalization of anguibactin (191). The energy for this internalization is provided by the ATPase FatE, which is encoded by a gene within the cluster for DHBA at a different locus than the other transport genes. A FatE mutant was still proficient in the uptake of the siderophore, while a double FvtE and FatE strain was not capable of internalizing the siderophore (192). The FvtE protein is involved in the uptake of vanchrobactin, a chromosomally encoded siderophore found in many *V. anguillarum* strains (193, 194). In strains harboring pJM1-type plasmids, genes of the vanchrobactin system, including the *fvtE* gene, are present on the chromosome, but this system is inactivated by an insertion in one of the vanchrobactin biosynthesis genes (195).

## Regulation

The best-studied region of pJM1-type plasmids is the so-called iron transport and biosynthesis (ITB) operon. This operon consists of six genes transcribed from a single promoter (161, 170). The first four genes, *fatD*, *C*, *B*, and *A*, are involved in iron transport, and the last two, *angR* and *angT*, in siderophore biosynthesis—hence the name.

Three plasmid-encoded factors act as positive regulators enhancing the expression of the ITB operon. One of these factors is the AngR protein, which is needed for the synthesis of the siderophore and encoded by a gene that is part of the ITB operon (196, 197). It was shown that the AngR protein enhances the expression of the ITB operon by 2.5-fold (198). The AngR polypeptide contains predicted helix-turn-helix and leucine zipper motifs common to DNA binding proteins and transcriptional regulators (199). In addition to AngR, another factor, called TAF, enhances gene expression of the ITB operon by about 4-fold (198). TAF stands for trans acting factor because it is located outside the ITB operon. If AngR- and TAF-positive regulation are combined, a 23-fold increase in expression is seen, indicating some synergistic/cooperative action between the two regulators (198). Besides the ITB operon promoter, AngR and TAF also regulate the expression of the *angN* gene (182).

The third factor is the anguibactin siderophore itself, which acts as a positive regulator of the ITB operon promoter (23).

Like most iron uptake systems, the iron uptake genes on the pJM1-type plasmid are regulated by the chromosomally encoded protein Fur (200–203). Fur binds to two loci upstream of the ITB operon promoter and bends the DNA by dimerization of the proteins at the two loci (202). Under iron-limiting conditions, the Fur protein dimer loses its iron and the dimer falls apart, abolishing DNA binding and thereby relieving repression (202). The regulation by Fur extends to other genes involved in anguibactin biosynthesis such as *angM* and *angN* (181, 182).

Besides this general regulator, the plasmid itself encodes two antisense RNAs that act as negative regulators of gene expression.

The first antisense RNA, RNAα, was discovered on the opposite strand of the *fatB* gene (203, 204). Tran-

scription of this antisense RNA requires the Fur protein, and under iron-replete conditions RNAα negatively regulates the *fatA* and *fatB* transcripts (203).

The second antisense RNA is located within the ITB operon, complementary to the *fatA* and *angR* genes (205). This RNA, called RNAβ, controls the differential gene expression between the transport and the biosynthesis genes within the ITB operon (205). In fact, although they are transcribed from the same promoter, the levels of *fatA* and *angR* differ by 17-fold (206). The difference is due to transcription termination between the *fatA* and *angR* genes that is mediated by RNAβ (206, 207). It has been proposed that RNAβ binds to the newly transcribed message, destabilizing the transcription complex, which leads to transcription termination (206). It is unknown if the two antisense RNAs control gene expression at loci other than their sense transcripts.

### Heterogeneity of the pJM1 Determinants

As already mentioned, the pJM1-type plasmids are highly similar. The six restriction length polymorphism patterns identified differ mainly in the number of insertion sequences (the ISV-A2 elements) present (161, 162). However, there are some minor changes at the nucleotide level that have major impacts. For instance, the DNA sequence of the *angR* gene differs by a single nucleotide between pJM1 plasmid from *V. anguillarum* 775 and the pJM1-type plasmid pJHC1 from *V. anguillarum* 531A (208, 209). Where a histidine is present at position 267 in the AngR protein encoded by the pJM1 plasmid, an asparagine is present in the AngR protein of pJHC1 (208). This nucleotide change results in higher anguibactin production in the 531A strain. The $LD_{50}$ values are similar for both bacteria, making it unclear what is the benefit for the bacterium to produce increased levels of siderophore (199).

When the three sequenced pJM1-type plasmids are aligned, only 16 nucleotide changes are revealed. Besides these single-nucleotide polymorphisms, the pJM1 plasmid has an extra 359-nucleotide HindIII fragment in the *angN* gene. The absence of this fragment in the M3 and pEIB1 plasmids results in the deletion of one of the two cyclization domains in AngN. Site-directed mutagenesis in the *angN* gene of pJM1 showed that at least one of the two Cy domains of AngN must be functional for anguibactin biosynthesis (182). However, one missing Cy domain reduced the production of anguibactin. The anguibactin-producing phenotypes of M3 and pEIB1 are not known. It would be of interest to see the effect of the deletion in AngN on anguibactin production, especially since the M3 plasmid and pEIB1 carry the point mutation in the *angR* gene that causes increased production of anguibactin in strain 531A. Another major change is the presence of an additional ISV-A2 element in the pEIB1 and M3 plasmids (163, 164).

While pJM1-type plasmids are found only in serogroup O1 strains, some genes are found outside this specific reservoir. The *fatA* and *fatD* genes have been identified on the chromosome of serotype O2a and NT4 *V. anguillarum* strains by PCR and DNA sequencing. These strains did not contain the *angR* gene (210).

A more striking discovery was the identification of an *angR* gene homolog in a whole-genome sequence of *Vibrio harveyi* (211). Further detailed analysis revealed that the chromosome of this bacterium encodes the complete ITB operon including the upstream *angM* gene and downstream genes *angH, angU*, and *angN*. In the case of *V. harveyi* the *angBG* gene is located in proximity to the cluster, while on the pJM1 plasmids the two clusters are located in noncontiguous regions (211).

## CONCLUDING REMARKS

The first identified plasmid harboring an iron uptake system was a ColV plasmid, which was followed soon after by the discovery of pJM1 (58, 157). Plasmid types encoding iron uptake systems are rare, and despite their concomitant discovery, ColV- and pJM1-type plasmids are quite different. The most striking difference is the presence of numerous iron and non-iron-related determinants on ColV against the single iron uptake system encoded on pJM1. The anguibactin system is the only plasmid trait identified so far on pJM1-type plasmids. Furthermore, the latter are restricted to serotype O1 *V. anguillarum* strains, while the ColV plasmids are more widespread even across genera (29, 41, 42, 51, 152). The restriction of pJM1-type plasmids could be the result of their inability to be transferred, whereas the ColV plasmids are conjugative. As a consequence, it is not surprising that, over 50 years after the first description of a plasmid-linked colicin V, ColV plasmids are still identified in isolates from human and animal infections (45, 117, 212, 213). Another important difference between the ColV- and pJM1-type plasmids is the recent association of the former with antibiotic-resistance determinants. Non-ColV plasmids harboring multidrug resistance and ColV virulence factors have been described in the past, but recently, a ColV plasmid encoding antibiotic resistance has been identified (212). The lack of such determinants on pJM1-type plasmids could be a consequence of lower levels of antibiotics in environments where *V. anguillarum* is present.

Alternatively, the limited interaction with other plasmids harboring resistance genes could have prevented the spread of multidrug resistance on pJM1-type plasmids.

The evolution of both plasmid types has most likely proceeded by the addition and deletion of large blocks of DNA. Entire systems are either absent or highly similar at the nucleotide level when present (214). Within this mosaic structure the iron uptake systems present are highly conserved across the members of the ColV and pJM1 types.

## ACKNOWLEDGMENTS

We thank Jorge H. Crosa, who passed away too soon. He not only formed the basis of both of our careers, but also of the plasmid-mediated iron uptake research.

Publication 5716 of the Netherlands Institute of Ecology (NIOO-KNAW).

Conflicts of interest: We disclose no conflicts.

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